cence spectra of authentic scopoletin and compound A were identical. The ir peaks were: 1570 and 1295 (vs), 1710, 1613, 1510, 1265, 1143, and 1020 (s), 1440, 1190, 925, and 865 (m), and 1380, 1224, 820, and 747 (w) cm^{-1}. Mass spectra were: *m/e* 50 (10%), 51 (18%), 65 (8%), 69 (36%), 79 (18%), 121 (17%), 149 (39%), 164 (21%), 177 (55%), 178 (6%), and 192 (100%), and corrected emission maximum, 380 nm (excitation, 365 nm). All of these observations show that compound A is scopoletin. The total scopoletin content of dried bract as estimated by tlc and fluorodensitometry was about 5 ppm.

Scopoletin occurs in cigarette smoke and tobacco (Yang *et al.*, 1958) and has been reported to increase greatly in fruits and vegetables infected by microorganisms (Hughes and Swain, 1960). Cotton plants affected with *Verticillium* wilt also apparently contain scopoletin (Caldwell *et al.*, 1966). Infection by the pathogen greatly enhances the production of scopoletin in the cotton plant, but physiological maturity of the plant determines the amount of scopoletin produced (Stith, 1969).

Steam treatment of raw cotton reduces byssinotic symptoms during carding (Merchant *et al.*, 1973). A similar treatment removes scopoletin from bract trash. Since phenols have been implicated in lung disease (Steinfeld, 1972), it should be determined whether scopoletin can contribute to byssinosis.

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A Fluorometric Method for the Determination of Residues of 1-Naphthaleneacetamide and 1-Naphthaleneacetic Acid on Apples

Robert Sigrist, Alfred Temperli, and Jakob Hurter*

1-Naphthaleneacetamide (NAAamide) and 1-naphthaleneacetic acid (NAA) were applied to apple trees to prevent fruit drop 4 weeks before harvest. Maximum residues detected by fluorescence spectrophotometry, on a whole fruit, fresh weight basis, did not exceed 0.049 ppm for NAAamide and 0.012 ppm for NAA. Compared to the toxicologically permitted amount of 1.5 ppm, the residues are small, but persistent. The sensitivities of the assay for NAAamide and NAA in apple extracts are 0.025 and 0.01 ppm, respectively. The recoveries for both growth regulators are 90-103%. No chromatographic steps are required using the assay procedure.

1-Naphthaleneacetamide (NAAamide) and 1-naphthaleneacetic acid (NAA) have been used for almost 30 years as growth regulators. Application shortly after blossom induces fruit thinning on apple trees and the same compounds applied 3-4 weeks before harvest prevent fruit drop. Replacement of these growth regulators with Sevin (*N*-methyl-1-naphthylcarbamate) as a fruit thinning agent failed, since the carbamate is extremely toxic to honeybees. Although NAAamide may cause less damage by the burning of leaves than NAA, it is less frequently used. Until now, sensitive methods of residue analysis for NAAamide have been unknown. For NAA, however, a previously reported uv absorption and colorimetric method could detect residues as low as 0.2-0.1 ppm in apples (Bache *et al.*, 1962). Similar procedures were developed for olives (Zweig *et al.*, 1964) and for pineapple (Young *et al.*, 1963) with a sensitivity of 0.03 ppm.

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To produce a sufficient biological effect, both growth regulators must be applied in a concentration of 20-100 ppm of the spraying solution. Consequently, residues below 0.1 ppm on apples may be expected. Therefore, it was necessary to develop a more sensitive detection procedure than those available. The fluorescence properties of NAAamide and NAA provide a specific method for the detection of NAA in microgram amounts (Hornstein, 1958). With this fact in mind, residue analyses of NAA have been conducted in citrus fruit (Jolliffe and Coggins, 1970) and, by a modified procedure, in Kinnow mandarin fruit (Coggins *et al.*, 1972) with a minimum detectable concentration of less than 0.1 ppm. In this paper a short and sensitive procedure is described for the determination of NAAamide and NAA on apples within 5-6 hr, omitting tedious chromatographic steps.

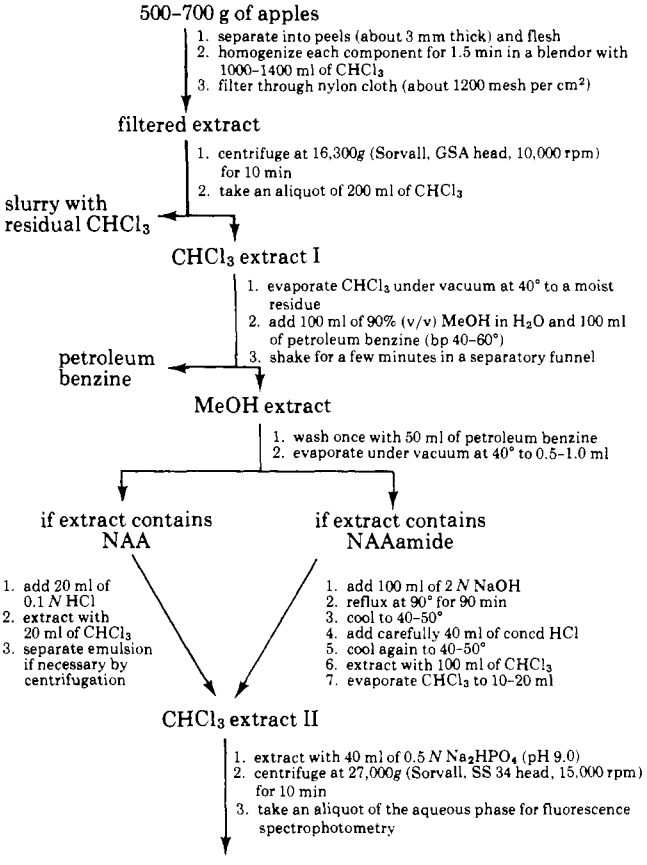
MATERIALS AND METHODS

Application of NAAamide and NAA to Apple Trees. To prevent fruit drop, a solution containing 10 g of

NAAamide or 2 g of NAA in 100 l. of water was sprayed on apple trees of the variety "Goldparmäne" 28 days before harvest. No analyses were conducted after treatment for fruit thinning, since we suppose the residues to be at least lower as the application took place 3 to 4 months before harvest.

Extraction and Identification of Growth Regulators from Fruit. After extraction, NAAamide was hydrolyzed and determined as NAA. The procedure for both growth regulators is outlined in the flow diagram of Chart I. Chloroform and petroleum benzine supplied by Merck, Darmstadt (West Germany) were of analytical grade. Chloroform was stabilized with 1.5% ethanol. For fluorometric determinations, a fluorescence spectrophotometer Model MPF-2A (Hitachi), connected with a Xenon power supply 150 (Perkin-Elmer), was used. The light source consisted of a xenon lamp type XBO 150/4 (Osram). For NAA, the maximum of exitation in 0.5 *N* Na_2HPO_4 (pH 9.0) was at 293 nm. The emission spectrum showed maxima at 325 and 335 nm. The 325-nm emission spectrum was selected for quantitative determinations. The instrument was calibrated for concentrations between 0.05 and 10.0 mg $l.^{-1}$. Within these limits, the deflection of the recorder was linear with concentration.

Chart I. Flow Diagram for the Extraction and Purification of NAAamide and NAA from Apples



RESULTS AND DISCUSSION

The minimum detectable amount of pure NAA was 0.01 mg $l.^{-1}$ in 0.5 *N* Na_2HPO_4 (pH 9.0) with a 1-cm light path. This value corresponds to the limit of detection in ethanol at 77°K using phosphorescence properties (Sanders and Winefordner, 1972). Following the extraction procedure of Chart I, 0.025 ppm of NAAamide and 0.01 ppm of NAA on apples can be identified. Because of the additional hydrolysis process, the minimum detectable level for NAAamide was somewhat higher. At residue concentrations below 0.1 ppm of NAA in the apple sample, the exitation maximum disappeared. The emission maximum at 325 nm, however, is still linear with concentrations as low as 0.01 ppm, but gradually loses its double peak shape. Independent of the residual amount, the efficiency of extraction for both growth regulators is 90–103%.

Table I shows the content of NAAamide and NAA detected in peels, in flesh, and in whole apples after treating trees against fruit drop. Each result represents the average value of four independent determinations. Residues of NAAamide in the whole fruit analyzed as NAA remain constant during the 28-day period. Results in flesh and on peels separately indicate the possibility that NAAamide is penetrating into the fruit. The data, however, cannot be considered as significant for this statement. Since NAA was applied at a concentration five times smaller than the amide, the residues on the whole fruit are at the minimum level of detection. The amounts on the peels remain constant; no residues were detectable in the flesh.

Based on toxicological investigations on rats (Anderson *et al.*, 1936; Lethco and Brouwer, 1966), mice (Höller *et al.*, 1958), dogs (Bernhard and Caflisch-Weill, 1949), and chick embryos (Dye *et al.*, 1944) an acceptable daily in-

Table I. Residues of NAAamide and NAA on Apples Treated 28 Days before Harvest

Growth regulator	Days after treatment	ppm		Whole fruit	
		Flesh	Peel	ppm	90% confidence limit
NAAamide (10 g in 100 l. of spray solution)	0	0.024	0.069	0.038	±0.021
	4	0.027	0.052	0.034	±0.013
	10	0.034	0.056	0.040	±0.021
	14	0.038	0.072	0.049	±0.021
	28	0.033	0.041	0.031	±0.013
NAA (2 g in 100 l. of spray solution)	0		0.025	0.012	±0.008
	4	Not	0.015	0.007	±0.006
	10	detectable	0.029	0.012	±0.005
	14		0.022	0.010	±0.006
	28		0.034	0.012	±0.007

Recovery of growth regulators from "spiked" control samples			
	ppm added	ppm found	Recovery, %
NAAamide	1.000	1.030	103
	0.500	0.490	98
	0.100	0.100	100
	0.025	0.023	94
NAA	0.050	0.051	102
	0.025	0.025	100
	0.010	0.009	90

take of 1.5 mg of NAAamide or NAA per person per day can be tolerated (Schlatter, 1973). Therefore, an intake of 1 kg of apples per day containing 1.5 ppm of NAAamide or NAA would represent an upper toxicological limit for adult persons. The detected amounts of NAAamide (0.049 ppm) and NAA (0.012 ppm) are far below this value. Since metabolic studies (Lethco and Brouwer, 1966) as well as carcinogenic investigations (Truhaut and Vermes, 1948; Innes *et al.*, 1969) have shown no indication that NAAamide or NAA may pose health hazards, there appears to be no objection to the agricultural use of these two growth regulators.

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A Method for Determination of Vicine in Plant Material and in Blood

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A colorimetric method for the determination of small quantities of vicine (2,6-diamino-4,5-dehydroxypyrimidine 5-(β-D-glucopyranoside)) in biological materials is described. Folin and Ciocalteu's phenol reagent is used as the chromatogen after removal of proteins, amino acids, and phenolic materials from extracts of plants and from blood plasma. The method makes possible the study of variations in vicine concentrations in fava beans, due to habitat and maturity. Its use will help elucidate the role of vicine as a causative factor in favism.

Favism is the term used for an acute hemolytic anemia which occurs in some people after the ingestion of the fava bean (*Vicia faba*). The condition exhibits a striking prevalence in the Mediterranean and Middle East area, but is rarely seen in other parts of the world although the bean is almost universally used as a cheap source of carbohydrate and protein. Susceptibility to fava bean is now accepted as being due to a glucose-6-phosphate dehydrogenase deficiency, but the causative agent is still not identified. One group of compounds thought likely to be implicated is the pyrimidine glycoside group found in considerable quantities in the fava bean (Mager *et al.*, 1965; Lin and Ling, 1962). The most common of these are vicine (2,6-diamino-4,5-dehydroxypyrimidine 5-(β-D-glucopyranoside)) and convicine (2,4,5-trihydroxy-6-aminopyrimidine 5-(β-D-glucopyranoside)), the former being present in greater amounts than the latter. The subject of favism has been recently reviewed (Mager *et al.*, 1969). To date, no method has been described to determine the concentration of these glycosides in small quantities of plant material or in plasma. This paper deals with such a method. Research on this subject was called for recently by the United Nations System (Protein Advisory Group Bulletin, 1973).

EXPERIMENTAL SECTION

Method. Vicine and convicine react with Folin and Ciocalteu's phenol reagent to give a blue color. Many other compounds react similarly with the reagent, and it is necessary to prepare a plant extract free from these. Extraction of the plant material with cold trichloroacetic acid solution gives a solution which is free from protein, nucleic acids, and other high molecular weight materials. Treatment of the extract with ether removes trichloroacetic acid together with fat soluble material. The resulting solution still contains tyrosine and dihydroxyphenylalanine, both of which give a strong reaction with phenol reagent, together with other water-soluble material. Treatment with copper carbonate forms copper complexes with amino acids and salts with acids. These are absorbed by alumina, together with other compounds. Under the conditions described, vicine and convicine are not absorbed by the alumina. After centrifugation the clear supernatant is used for the color reaction.

Reagents. *Vicine and Convicine.* These glycosides are not available commercially and were prepared from fresh fava beans by a published method (Bendich and Clements, 1953).

Procedure. *Standard Curve.* Vicine was dissolved in water to given concentrations of 5, 10, 15, 20, 25, 50, 75, and 100 μg/ml. One milliliter of each of the solutions and a water blank were mixed with 1 ml of phenol reagent (Folin and Ciocalteu, 1927) which had been diluted 30 times with water; 2 ml of aqueous 20% sodium carbonate was then added to each and the mixture was shaken for a few seconds. The mixture was left at room temperature for 30 min and its density was then read at a wavelength of 650 nm.

Plant Material. A mixture of 1 g of finely divided plant material, either fresh or dry, was shaken with 10 ml of

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